Cytotechnology (2008) 57:61–66
DOI 10.1007/s10616-008-9130-7

SI:HETEROLOGOUS GENE EXPRESSION

Bioreactor culture of recombinant *Drosophila melanogaster* S2 cells: characterization of metabolic features related to cell growth and production of the rabies virus glycoprotein

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Received: 12 November 2007 / Accepted: 21 January 2008 / Published online: 19 February 2008

Abstract Although several reports have been published on recombinant protein expression using *Drosophila* cells, information on their metabolism and growth in vitro is relatively scarce. In the present study, we have analyzed the growth and metabolism of transfected S2 cells (S2AcRVGP) in bioreactor cultures with serum-free medium Sf900 II, to evaluate its potential for mass production of a rabies virus glycoprotein (RVGP). Cells were cultured in a 3 l-stirred-tank bioreactor at 28 °C with pH controlled at 6.2 and dissolved oxygen at 50% air saturation. The cells attained a specific growth rate and maximum cell density as high as 0.084 h^{-1} and 2.3 × 10^7 cell ml^{-1}, respectively. The main substrates consumed during this rapid growth phase were glucose, glutamine and proline. An atypical accumulation of ammonia and alanine was observed in the culture medium, up to 62 mM and 47 mM, respectively, but lactate was produced in low levels. After exhaustion of glutamine and proline as energy sources, alanine was consumed and production of ammonia increased. The production of recombinant RVGP reached concentrations as high as 178 μg l^{-1}. Premature exhaustion of glutamine, serine and cysteine could be related to degradation of the recombinant glycoprotein. In general, the results demonstrated that S2AcRVGP can be considered an effective vehicle for large-scale recombinant glycoprotein expression and that several critical factors of the bioprocess could be optimized to increase the quality and productivity of the RVGP.

Keywords Bioreactors · *Drosophila melanogaster* · Growth · Metabolism · Rabies virus glycoprotein · Recombinant S2 cells

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Introduction

In the last six years the *Drosophila* S2 protein expression system has been successfully used to produce a series of complex recombinant proteins (Kim et al 2008; Johansson et al 2007; Lim and Cha 2006; Cha et al 2005; Perret et al 2003; Cho et al 2004; Valle et al 2001). An analysis of the literature reveals that the major concern of researchers dealing with S2 cells has been the expression of high levels of the recombinant proteins, while there has little emphasis on collecting information about the influence of key factors such as cell metabolism or the composition of the medium on cell growth and biosynthesis of the recombinant protein. In this scenario, increasing our knowledge of the performance of S2 cells would be of use in the control and optimization of culture

parameters, so as to meet the needs of the cells and maximize the yield and quality of the end product.

In this study, some important features of the culture of recombinant *Drosophila* S2 cells in bioreactor expressing the rabies virus glycoprotein (RVGP), which influence strongly the performance of the bioprocess have been identified and quantified in order to improve the large-scale RVGP production.

Materials and methods

Cells and culture conditions

Recombinant S2 cells (S2AcRVGP) for production of RVGP, transfected according to Yokomizo et al. (2007), were used. The cells were cultured at 28 °C in serum-free Sf900 II medium (Gibco®/Invitrogen Life Technologies, Carlsbad, CA) in a 3L Bioflo 110 bioreactor (New Brunswick Scientific, USA) with 1 l of working volume, equipped with a pitched-blade impeller, pH being controlled at 6.20 through the addition of NaOH 0.5 M and H_2SO_4 8% and dissolved oxygen (DO) at 50% air saturation, oxygenation carried out by diffusion through a silicon membrane (7.84 m). The bioreactor was initially seeded at 28 °C and 5×10^5 cell ml^{-1} with cells taken from a mid-exponential 500 ml spinner flask (Wheaton®) culture at 100 rpm and 28 °C.

For the evaluation of S2AcRVGP cell performance in serum-free Sf900 II medium several experiments were run in the bioreactor under four different culture conditions, as described in Table 1.

Analytical methods

Cell counts

The total cell density was determined with a hemacytometer and the viability by the trypan blue exclusion method (Doyle and Griffths 1998). The lysed cell density was estimated from the activity of LDH, released from these cells into the supernatant, employing an enzyme assay kit (Tox−7) from Sigma Aldrich Co.

Nutrients and metabolite analysis

The concentrations of glucose, lactate and glutamine in the culture supernatants were determined enzymatically with a YSI Biochemical Analyzer (model 2700) after sample centrifugation at 200 *g* for 5 min. Amino acid concentrations in the culture supernatants were analyzed by the Pico-tag system, using a

Table 1 Bioreactor experiments to analyze the growth and metabolism of S2AcRVGP cells in Sf900 II medium

Expt.	Description	Observations
Bio-1	Batch mode, with increasing stirring (15 rpm/day), from 100 to 240 rpm. Medium Sf900 II	To evaluate the possibility of using higher stirring speeds than in the previous report (Swiech et al. 2008), in order to improve homogenization of the bubble-free culture in the bioreactor without damaging the cells.
Bio-2	Batch mode, with increasing stirring (15 rpm/day), from 100 to 240 rpm. Medium Sf900 II supplemented with 8.7 mM Pro	In order to analyze the effects of proline as energy source, on growth and expression of RVGP in a homogenized bioreactor.
Bio-3[a]	Fed-batch mode: F = 4 ml h^{-1}, V_0 = 800 ml and V_f = 1,400 ml. Accelerating stirring, starting at 100 rpm and rising 8 rpm /day for the first 4 days, 30 rpm/day for the last two days. Feed medium: Sf900 II + 15.6 mM Pro; 0.6 mM Ser; 8.6 mM Gln, 0.005 mM Cys	The feeding strategy was aimed at gradual addition of the aminoacids prematurely depleted in batch mode. When Cys was added at the higher concentration of 1 mM, cell growth was strongly inhibited (data not shown). The initial stirring speed was low to avoid shear damage to cells due to excessive volumetric energy input in a small volume.
Bio-4	Batch mode, constant stirring rate at 150 rpm. Medium Sf-900 II	To evaluate the use of an intermediate, constant stirring rate, sufficiently high to prevent poor mixing but not too high, to avoid shear damage to cells.

[a] F, volumetric flow rate; V_0, initial bioreactor volume; V_f, final bioreactor volume

reverse-phase HPLC column (Waters Sweden, Sollentuna, Sweden) (Bidlingmeyer et al. 1985). Ammonia was determined through a 95–12 Orion Probe Analyzer (Orion), and a SA720 Procyon potentiometer.

RVGP concentration

The RVGP is a transmembrane protein present in cell lysates which were evaluated by ELISA (enzyme-linked immunosorbent assay) with immunoglobulin (IgG) against rabies glycoprotein (RVGP), purified from the serum of rabbits immunized with purified RVGP from rabies virus (Pasteur lineage) propagated on Vero cells, as a cover antibody. The cover antibody labeled with peroxidase was used to detect bound RVGP antigen as described by Astray et al. (2008).

Results and discussion

Cell growth and metabolism

As can be observed in Fig. 1a and in Table 2 the growth parameters of S2AcRVGP cell are higher than reported in cultures of lepidopteran cell lines (Schmid 1996) and higher than obtained in S2AcRVGP cell culture in Schott flasks (Yokomizo et al 2007) and bioreactor without oxygen control (Swiech et al. 2008). Even so, some modifications seem to be necessary for optimization of S2 cell growth and production of the RVGP, as will be shown ahead.

The three more consumed substrates during cell growth were glucose, glutamine and proline, as can be seen by Fig. 1b, c. The first two substrates are known to play key roles as energy sources and as precursors for biomass synthesis in animal cell culture. On the other hand, the role of proline as an energy source has been reported in a previous article to be a peculiar metabolic feature of some types of insects (Swiech et al 2008). Comparison of experiments Bio-1 and Bio-2 reveals a strong dependence of the specific growth rate of S2AvRVGP cells on the concentration of proline, being for that reason considered an energy source. With the addition of 8.7 mM of proline, Bio-2 reached $\mu_{max} = 0.084\ h^{-1}$, almost twice the value attained in experiment Bio-1 ($0.048\ h^{-1}$). The substantial difference in μ_{max} between the two experiments could be attributed to the higher quantity of this amino acid in Bio-2. The addition of proline and glutamine in experiment Bio-3 favored the growth in terms of maximum cell concentration but not in specific growth rate because the concentrations of these nutrients were maintained at levels similar to those of fresh Sf900 II medium by gradual feeding of the batch.

The experiment Bio-4, with constant stirring at 150 rpm, did not show any lag phase in cell growth as response to higher shear stress, suggesting that the stirring at around 100 rpm during the first hours of the other three experiments may have resulted in a heterogeneous and oxygen-transfer limited environment. In such favorable conditions the maximum cell density reached in experiment Bio-4 was 2.3×10^7 cell ml^{-1}. As the cell death rate in this experiment was still as low as in the others, the stirring rate of 150 rpm can be recommended as a minimum for S2 cell culture in the bioreactor set-up described.

Regarding glutamine consumption in the four experiments, it is clear in the growth curves that when the concentration fell below about 2 mM, a significant reduction in the growth rates ocurred. Additionally, after the exhaustion of glutamine, similarly to what happens in mammalian cell cultures, viable cell growth ceased and the cell density started to decline.

The low values of LDH activity assayed throughout the whole culture, including the declining phase, suggest that little cell lysis occurred. The high values of μ_{max} ($0.084\ h^{-1}$) in experiment Bio-2 and of X_{Vmax} (2.3×10^7 cells.ml^{-1}) in experiment Bio-4 provide an illustration picture of the potential for growth of *Drosophila melanogaster* S2 cells in stirred tank bioreactors.

Despite the high concentrations of amino acid of medium Sf900 II, some of them (glutamine, proline, cysteine and serine), were exhausted prematurely during the cultures. Proline was an amino acid also utilized as an energy source, when added in experiment Bio-2, it increased the μ_{max} to $0.084\ h^{-1}$, a value rarely seen in animal cell culture. The main products of glutamine and proline metabolism were ammonia and alanine. Alanine was produced in considerable amounts in all the experiments, reaching levels as high as 47 mM in experiment Bio-2, in which Sf900 II medium was supplemented with

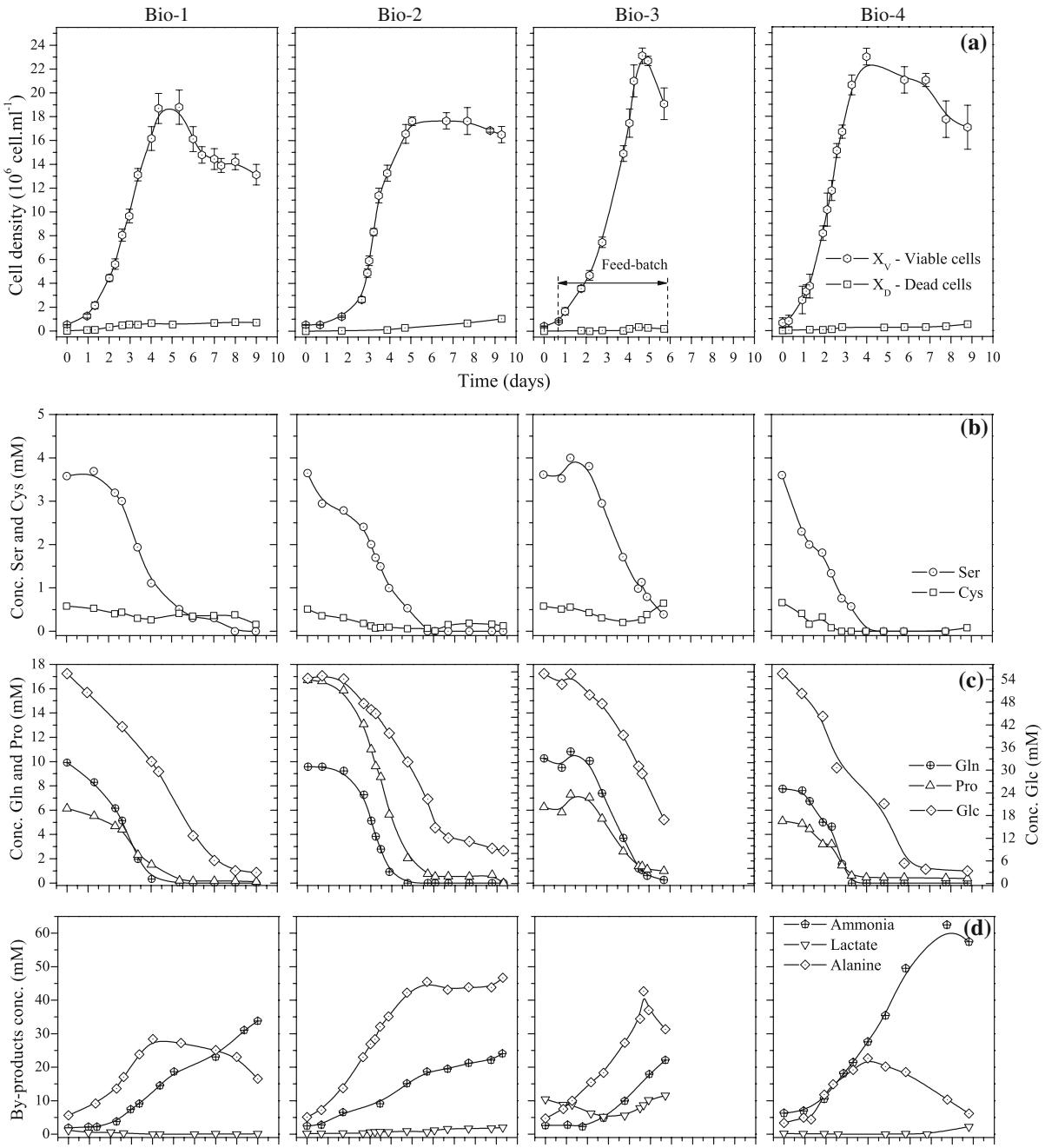

Fig. 1 Results of growth and metabolism of S2AcRVGP cells cultured in a stirred tank bioreactor with Sf900 II medium at 28 °C, DO = 50% and pH = 6.20. The bars represent the standard error

8.7 mM proline. Most of the serine and cysteine was consumed for protein synthesis, as will be discussed later. Other amino acids, especially alanine, asparagine and glutamate, were consumed only after exhaustion of glutamine and proline, possibly as new energy sources. This amino acid degradation should be responsible for the high accumulation of ammonia after glutamine exhaustion. In experiment Bio-4, in which the highest cell density was produced, the level of ammonia reached 62 mM. Until

Table 2 Summary of parameters for S2AcRVGP cell growth in the experiments in bioreactor described in Fig. 1

	μ_{max} (h^{-1})	X_{Vmax} (cell ml^{-1})
Bio-1	0.048 ± 0.003 (R = 0.993)	1.88×10^7
Bio-2	0.084 ± 0.006 (R = 0.995)	1.76×10^7
Bio-3	0.035 ± 0.001 (R = 0.999)	2.31×10^7
Bio-4	0.059 ± 0.005 (R = 0.991)	2.30×10^7

R, correlation coefficient of semilog plot of cell density vs. time in the exponential phase

now, such a high ammonia concentration has never been reported in animal cell culture, indicating that these cells are very resistant to high levels of ammonia. Interestingly, Borash et al (2000) have shown that the insect *Drosophila melanogaster* also has a high tolerance to ammonia. Lactate was present in very low concentrations in all the experiments.

The sequential behavior of S2AcRVGP cells, involving glutamine degradation and alanine accumulation followed by a switch to consumption of amino acids, including alanine, marked by high production of ammonia under glutamine limitation, is somewhat similar to the behavior of Sf-9 cells under glucose limitation observed by Öhman et al (1995). The differences that exist may be due to the existence of different networks for glucose and glutamine metabolisms in *Spodoptera frugiperda* and *Drosophila melanogaster* cells.

RVGP production

The time course of the biosynthesis of the glycoprotein RVGP during the four experiments in bioreactor can be seen in Fig. 2. As expected, being the RVGP a membrane protein, its biosynthesis can be growth associated. The cell-lysate expression levels reached values up to 178 μg l^{-1}, as can be seen more precisely in Table 3. In experiment Bio-2, the production of RVGP was very low because of the addition of proline, which favored the utilization of the metabolic machinery for cell proliferation. Although production was very high in the other experiments, in experiments Bio-1 and Bio-4 an accentuated drop in RVGP concentration is seen after approximately 5 days of culture. This drop reflects the degradation of this protein, a phenomenon that may be explained by the depletion of glutamine,

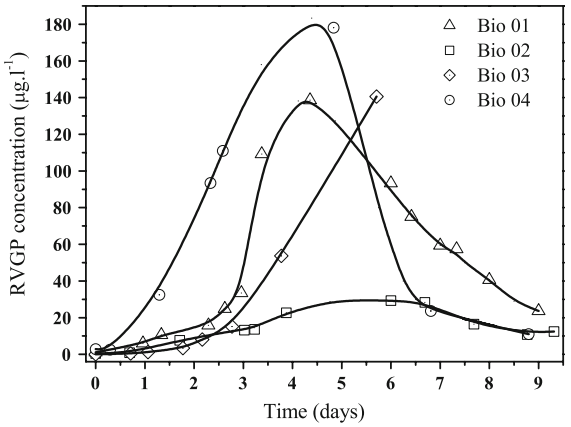

Fig. 2 RVGP production in the four bioreactor experiments with S2AcRVGP cells

Table 3 RVGP production parameters in the four bioreactor experiments with S2AcRVGP cells

	C_{max}^{RVGP}(μg l^{-1})	P_{max}^{RVGP} (μg l^{-1} h^{-1})
Bio-1	138.7	1.33
Bio-2	29.4	0.20
Bio-3	140.5	1.03
Bio-4	178.1	1.54

serine and cysteine, two of which are directly involved in protein structure folding and stabilization. The serine participates intensively in protein processing through glycosylation of its side chains serving, one purpose of which is to assist in proper folding and to protect the biomolecule from protease degradation (Jentoft 1990). On the other hand, cysteine is well known for its role in the formation of covalent sulfur bridges that play a crucial role in the stabilization of proteins (Freedman 1984). Strong evidence in favor of this hypothesis is the result obtained in experiment Bio-3, in which the continuous feeding of the amino acids prevented RVGP depletion and, therefore, any degradation of the glycoprotein. It is evident from these results that the medium Sf900 II has to be fed with these three amino acids in order to accomplish optimal productivity of a stable recombinant RVGP. It will also be necessary to define the feeding strategies, coordinated with the addition of proline, that allow non-limited and robust cell growth.

Conclusions

Taken together, the results presented here show that S2AcRVGP cells can be successfully used in large-scale cultures in a serum-free medium for recombinant RVGP production. The cells achieved high specific growth rates and cell densities, as well as a notable tolerance to the adverse conditions normally present in bioreactor cell cultures. In addition, S2AcRVGP cells were able to synthesize fair quantities of RVGP and represent an attractive alternative source for its production. This study also identified some critical bioprocess factors that still need to be optimized, in the formulation of culture medium and the operating strategy of the stirred-tank bioreactor.

Acknowledgements This work was supported in part by grants from FAPESP (02/09482-3, 01/09038-3, 01/08914-4), Fundação Butantan and CNPq (141099/2003-8).

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